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Short communication

Changes in antiviral susceptibility to entry inhibitors and endocytic uptake of dengue-2 virus serially passaged in Vero or C6/36 cells

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ABSTRACT

The aim of the present study was to analyze the influence of virus origin, mammalian or mosquito cell-derived, on antiviral susceptibility of DENV-2 to entry inhibitors and the association of this effect with any alteration in the mode of entry into the cell. To this end, ten serial passages of DENV-2 were performed in mosquito C6/36 cells or monkey Vero cells and the antiviral susceptibility of each virus passage to sulfated polysaccharides (SPs), like heparin and carrageenans, was evaluated by a virus plaque reduction assay. After serial passaging in Vero cells, DENV-2 became increasingly resistant to SP inhibition whereas the antiviral susceptibility was not altered in virus propagated in C6/36 cells. The change in antiviral susceptibility was associated to a differential mode of entry into the host cell. The route of endocytic entry for productive Vero cell infection was altered from a non-classical clathrin independent pathway for C6/36-grown virus to a clathrin-mediated endocytosis when the virus was serially propagated in Vero cells. Our results show the impact of the cellular system used for successive propagation of DENV on the initial interaction between the host cell and the virion in the next round of infection and the relevant consequences it might have during the in vitro evaluation of entry inhibitors.

Dengue virus (DENV), a member of the genus *Flavivirus* in the family *Flaviviridae*, is at present the most widespread arbovirus in the world (Guzmán et al., 2010). The World Health Organization estimates that 50–100 million infections occur each year, though new appraisals indicate that apparent and inapparent infections would reach the number of 350 million per year (Bhatt et al., 2013). Four serotypes of DENV, DENV-1 to DENV-4, co-circulate in tropical and subtropical regions of Asia, Africa and America by human transmission through the bite of an infected mosquito from the species *Aedes aegypti* and *Aedes albopictus*. All serotypes may cause an inapparent infection, a mild illness known as dengue fever or the severe dengue hemorrhagic fever/dengue shock syndrome (Halstead, 2007). However, no specific chemotherapy neither vaccine for dengue is currently available and treatment is based only in supportive care.

Different types of sulfated polysaccharides (SPs) were found to be potent and selective inhibitors of DENV infection. The antiviral activity of SP against DENV is dependent on virus serotype and the host cell used for the antiviral assay. For virus plaque, focus or yield reduction tests performed in Vero (Pujol et al., 2012; Talarico et al., 2005), BHK-21 (Hidari et al., 2008; Lin et al., 2002) HepG2 (Talarico and Damonte, 2007) or LLC-MK2 cells (Ichiyama et al., 2013) it has been reported that DENV-2 is the most susceptible serotype to SP inhibition, whereas DENV-3, DENV-4 and DENV-1, in this order, require higher compound concentration for an effective action. It was demonstrated that the antiviral action of SP against DENV-2 is exerted by a blockade of virus adsorption and penetration (Ichiyama et al., 2013; Talarico and Damonte, 2007), due to the structural similarities with the heparan sulfate (HS) residues of membrane proteoglycans, proposed as putative initial cell receptor for DENV-2 (Chen et al., 1997; Dalrymple and Mackow, 2011; Germi et al., 2002; Hilgard and Stockert, 2000). With respect to the host cell, the efficacy of SPs against DENV-2 was similar in the cited mammalian cells, but they were almost inactive in mosquito C6/36 cells (Talarico et al., 2005).

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In the above-mentioned studies, the working viral stocks used to analyze antiviral activity were propagated in mosquito C6/36 cells. In nature, the first round of infection in a human being is with virus derived from mosquito cells, and subsequent infections in the same host occur with human-derived virus as well as the next round of transmission from the human host to a new mosquito vector. Differences in the source of virus may affect the interaction between the virus and the host cell and, likely, the antiviral susceptibility. The effect of viral source has been examined for some particular DENV–host interactions such as virulence/attenuation, replication ability and receptor affinity (Añez et al., 2009; Hacker et al., 2009; Lee et al., 2006, 2011; Prestwood et al., 2008). The aim of the present study was to evaluate the impact of virus origin, mammalian or mosquito cell-derived, on antiviral susceptibility of DENV-2 to SP and the association of this effect with any alteration in the mode of entry into the cell.

The C6/36 mosquito cell line was cultured at 33 °C in L-15 medium (Leibovitz) (GIBCO) supplemented with 0.3% tryptose phosphate broth, 0.02% glutamine, 1% minimum essential medium (MEM) non-essential amino acids solution and 5% fetal calf serum whereas Vero monkey cells were grown at 37 °C in Eagle's MEM (GIBCO) supplemented with 5% calf serum. Maintenance medium (MM) consisted of MEM containing 1.5% calf serum. For cellular virus adaptation, ten serial passages of the original stock of DENV-2 strain NGC (provided by Dr. A.S. Mistchenko, Hospital de Niños Dr. Ricardo Gutiérrez, Buenos Aires, Argentina), usually produced in C6/36 cells and designed DENV-2 V0, were carried out either solely in Vero (named V for Vero cells and the corresponding passage number) or C6/36 cells (named M for mosquito cells and passage number). To this end, Vero or C6/36 cell monolayers grown in 6-well microplates were infected with DENV-2 at an m.o.i. of 0.1 PFU/cell. Supernatants were collected at 4–7 days post-infection (p.i.) and titrated by plaque formation in Vero cells. The supernatant with the highest virus titer was selected to perform the next passage and serial passages were continued in this manner. Two series of independent sequential cell passages were performed and analyzed separately.

First, the antiviral activity of heparin (Sigma–Aldrich) against C6/36- or Vero-adapted DENV-2 was evaluated by a virus plaque reduction assay in the presence of serial two-fold compound concentration as previously described (Talarico et al., 2005). A dose dependent inhibitory response was observed for the original virus and the four initial passages in Vero cells, as shown for DENV-2 V0 and DENV-4 V4 in Fig. 1. However, for the virus serially propagated in Vero cells, the susceptibility to heparin decreased dramatically between passages 4 and 5, and no inhibition in plaque number was detected for DENV-2 V10 (Fig. 1). By contrast, the virus continuously propagated in mosquito cells DENV-2 M10 presented a susceptibility to heparin very similar to DENV-2 V0 (Fig. 1).

The variable resistance to heparin after DENV-2 passage in Vero or C6/36 cells was clearly evidenced when the values of effective concentration 50% (EC_{50}), the compound concentration required to reduce plaque number by 50%, were calculated from data shown in Fig. 1. No inhibition of DENV-2 V10 multiplication was observed up to the highest concentration tested of heparin (50 μg/ml) whereas EC_{50} value for the original DENV-2 V0 was 2.6 μM (Table 1). A similar trend of change from DENV-2 V0 to DENV-2 V10 was observed when the antiviral susceptibility was evaluated against other types of SP exhibiting more potent activity against DENV-2 such as λ- or ι-carrageenan (Sigma–Aldrich). The values of EC_{50} against carrageenans corresponding to DENV-2 continuously replicated in Vero cells were always higher than the EC_{50} of the original DENV-2 V0, especially in the case of ι-carrageenan (Table 1). Differently from DENV-2 V10, DENV-2 M10 did not show significant increased resistance to any SP treatment (Table 1).

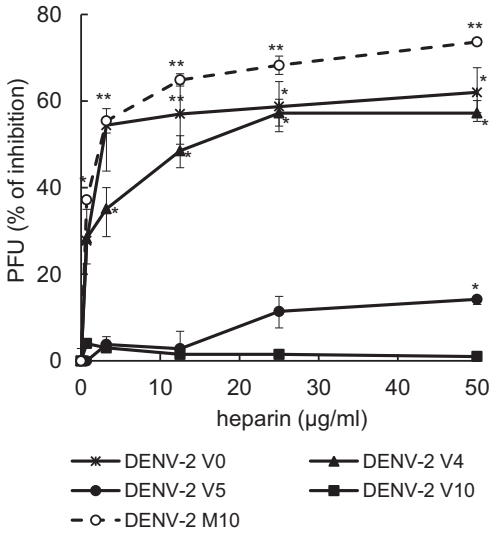

Fig. 1. Effect of heparin on DENV-2 infection. Vero cells were treated with serial twofold concentrations of heparin during 1 h and then infected with about 50 PFU/well of each DENV-2 passage in the presence of heparin. After adsorption, residual inoculum was replaced by MM containing methylcellulose. At 6 days p.i. cells were fixed with 10% formaldehyde, stained with 1% crystal violet and plaques were counted. Results are expressed as % inhibition of plaque number in compound-treated cultures compared to untreated ones. Each value is the mean of duplicate assays ± SD. Student's *t*-test was used to determine statistical significance between treated and control infected cells for each passage. Asterisks indicate *P* values in each dose–response curve (*$P \leq 0.05$; **$P \leq 0.001$).

A noticeable differential susceptibility to heparin and carrageenans was also reported among DENV serotypes (Talarico and Damonte, 2007). Interestingly, DENV-2 and DENV-1, the most susceptible and resistant serotypes to SP, respectively (Ichiyama et al., 2013; Talarico et al., 2005) use a different pathway for infectious entry into Vero cells: DENV-1 utilizes a clathrin-dependent pathway of endocytosis whereas the entry of DENV-2 occurs by a non-classical endocytic route independent of clathrin and caveolae, but dependent on dynamin (Acosta et al., 2009). Thus, we decided to assay the route of entry into Vero cells, regarding to clathrin-dependence, of the DENV-2 suspensions serially passaged in Vero or C6/36 cells to find the basis of their differential susceptibility to SP.

The involvement of clathrin-mediated pathway in the entry of DENV-2 after each passage was addressed by determining the effect of chlorpromazine (CPZ), a pharmacological inhibitor of this endocytic route (Wang et al., 1993). Vero cells were treated with 50 μM CPZ, the maximum noncytotoxic concentration, only for 2 h before infection and during the first hour of infection with each DENV-2 passage when entry takes place. Under these treatment conditions the clathrin-mediated endocytic pathway is effectively blocked, as assessed by transferrin internalization assays (data not shown). Extracellular virus yields were determined at 48 h p.i. by plaque formation in Vero cells. As previously reported for the original DENV-2 V0 (Acosta et al., 2009), CPZ treatment did not produce any inhibitory effect on infection of Vero cells with initial passages of DENV-2 V0 in Vero cells (DENV-2 V1 to V3), but surprisingly, after 4 passages a 50% reduction in virus yield was observed after CPZ treatment as compared to untreated control (Fig. 2A). The inhibitory activity of CPZ against DENV-2 increased with Vero cell passage number and after 7 passages the inhibition in virus yield remained over 90% with respect to control non-CPZ treated cells. The parallel 10 serial passages of DENV-2 V0 in mosquito C6/36 cells resulted in the generation of virus populations without increased susceptibility to CPZ treatment in Vero cells. Conversely, a tendency to produce higher amounts of